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THE CARBON ASSIMILATION OF PENICILLIUM

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 108

HEINRICH HASSELBRING

Since PASTEUR first showed that the lower fungi (yeasts in this case) could be grown on nutrient solutions containing organic substances other than those derived from plant and animal tissues, a large number of organic compounds have been examined with regard to their nutritive value for molds. It has been found that the most varied and widely different compounds are suitable in one case or another for supplying carbon and nitrogen to those organisms. All the fungi, however, cannot utilize the various compounds with equal facility. Thus, while *Penicillium* is almost omnivorous, thriving on alcohol, organic acids, sugars, and many other substances, *Mucor racemosus* and *M. javanicus*, according to WEHMER,² and yeast, according to LAURENT,³ do not assimilate alcohol. In like manner, two very closely related compounds, such as the optically active modifications of tartaric acid, as well as chemical isomers without an asymmetric carbon atom,⁴ may possess very different nutrient value.

The knowledge that a great number of organic compounds of the most varied structure could supply fungi with the necessary food and energy for all of their activities, soon led to attempts to establish some relation between the structure of organic compounds and their nutritive value. One of the first of these attempts was that of NÄGELI,⁵ who came to the conclusion, as the result of the study of a large number of substances, that food value depended upon the specific linkage of

¹ PASTEUR, L., Les corpuscules organisés qui existent dans l'atmosphère. Ann. Chim. et Phys. III. 64:5-110. 1862.

² WEHMER, C., Ueber das Verhalten der *Mucor*-Arten gegen verdünnten Alkohol. Ber. Deutsch. Bot. Gesells. 23:216, 217. 1905.

³ LAURENT, E., Nutrition hydrocarbonée et formation de glycogène chez la levure de bière. Ann. Inst. Pasteur 3:113-125. 1889.

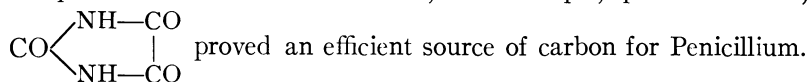
⁴ BUCHNER, E., Notiz aus der Gährungschemie. Ber. Deutsch. Chem. Gesells. 25:1161-1163. 1892.

WEHMER, C., Beiträge zur Kenntnis einheimischer Pilze 87-104. 1903.

⁵ NÄGELI, C., Ernährung der niederen Pilze durch Kohlenstoff- und Stickstoffverbindungen. Bot. Mitth. 3:395-485. 1881.

certain atomic groups. According to NÄGELI, carbon is assimilated from the groups $=CH_2$ and $\equiv CH$, but in the latter case only when it occurs in a chain of two or more C-atoms to which H is bound. Benzoic acid is assimilated, but formic acid is not. On the other hand, carbon is not assimilated when it is directly united only with O or N but not with H. Accordingly, the CN group, oxalic acid, urea, and similar substances, are not suitable sources of carbon.

Subsequent researches showed that the conclusions of NÄGELI were no longer tenable, since it was found that the molds were not restricted to such specific groupings as he supposed. REINKE⁶ greatly extended the list of substances which could be assimilated, and showed that some of the particular groupings which NÄGELI excepted could be utilized. Thus, for example, parabanic acid,



DIAKONOW⁷ demonstrated that carbon from urea and from formic acid could also be utilized by this fungus.

All of these facts indicate that no general relation can at present be established between the atomic structure of a substance and its food value. The mechanism of assimilation probably differs with each individual case and is as much dependent upon the nature of the plant as upon the chemical reactions of the compounds used; for, as already stated, every substance that has nutritive value for one plant will not serve as a food for all plants.

It was with a view of gaining, if possible, some knowledge of the complex problem of the chemistry of the assimilation of some of the simpler compounds that the work reported here was undertaken. It was thought that by studying the effect of a number of related compounds on the growth of the mold fungi, and noting the variations caused by different stimulating agents, it would be possible to gain some knowledge of the probable chemical reactions by which nutrient substances combine with the constituents of the living cell.

This proved to be a most complex problem, whose solution is made

⁶ REINKE, J., *Unters. Bot. Lab. Göttingen. (Rev. in Bot. Zeit. 41:551. 1883, and Just's Bot. Jahresb. 11:55. 1883.)*

⁷ DIAKONOW, N. W., *Organische Substanz als Nährsubstanz. Ber. Deutsch. Bot. Gesells. 5:380-387. 1887.*

more difficult on account of the narrow limits of experimentation to which living organisms can be subjected. Although no finally satisfactory conclusions have been reached, a few series of cultures have been worked out with great care. As I am for the present compelled by circumstances to discontinue the work, it is desirable to present the results thus far obtained, with a few final remarks on their possible significance.

METHODS

The form selected for this work was a strain of *Penicillium glaucum*. A number of other molds were tried, but were rejected because they did not thrive well under the conditions of the experiments. *Penicillium*, which has been used in innumerable physiological investigations, is especially suitable for this work on account of its omnivorous habit, and because many compounds have been studied with regard to their nutritive value for this fungus. The same strain of the mold was used throughout. The stock-cultures were always grown on sterilized bean-stems, so as to avoid any possible temporary influence of the substratum on the strain.

The culture medium used was a solution of the necessary inorganic salts, of the purest grades obtainable in the market. Except where specially noted, the culture fluid always contained the mineral salts in the same concentration, namely, 1^{gm} NH_4NO_3 , 0.5^{gm} KH_2PO_4 , and 0.25^{gm} MgSO_4 , per 100°C . Potassium chlorid was added in some cases, and the concentration of the magnesium salt was varied in others. These changes are noted in the respective tables. It was the purpose in this work to have the mineral solution alike in all the cultures in order to make the different series exactly comparable. This was carried out, except where special problems occurred which demanded a change from the standard.

As a source of carbon the following substances were used: alcohol ($\text{C}_2\text{H}_5\text{OH}$), potassium ethyl sulfate ($\text{C}_2\text{H}_5\text{KSO}_4$), ethyl nitrate ($\text{C}_2\text{H}_5\text{NO}_3$), ethyl acetate ($\text{CH}_3\text{COOC}_2\text{H}_5$), potassium acetate (CH_3COOK), and acetic acid (CH_3COOH). These compounds are all closely related, and have certain radicles in common. The combination of those radicles with various groups has a definite effect upon the mode of reaction of the compounds. By a comparative study of a large number of compounds of this nature with regard

to their assimilation by the molds, it was deemed possible to throw some light on the mechanism of their assimilation. The simpler compounds are most suitable for studies of this nature; first, because their reactions are well understood, and second, because it is known that the complex compounds, such as polysaccharides, glucosides, and certainly in some cases even the hexoses, undergo decomposition before they are taken into the cell.⁸ The solution of the mode of assimilation of the simpler compounds will therefore enable us to approach an interpretation of this process with the more complex compounds.

Cultures were made in 200^{cc} flasks, of the Erlenmeyer form, loosely stoppered with cotton to permit the free exchange of air. In each flask 50^{cc} of the culture medium were used. The mineral solution was first put into the flasks and sterilized for 20 minutes in flowing steam, after which the organic solution was added. The concentration of all the substances given is the final concentration after the addition of the organic solution, without allowing, however, for error caused by evaporation in the sterilizer, which averaged a little less than 0.5^{gm} per flask. In cases where acid was added the error caused by this addition was also not considered. These two errors compensated each other. Moreover, it was found that much greater differences than these produced no effect on the growth of the fungus, and a variation in the concentration is furthermore unavoidable on account of a slight evaporation in the incubator, which could not be prevented. The cultures were grown in an electrically regulated and electrically heated incubator, which permitted the maintenance of a constant temperature of 28° C. The duration of the cultures, except where noted, was 10 days.

Considerable effort was made to work out the best method for inoculating the flasks. On account of some observations of DUCLAUX,⁹ indicating that some media which were suitable for nourishing

⁸ PURIEWITSCH, K., Ueber die Spaltung der Glycoside durch die Schimmelpilze. *Ber. Deutsch. Bot. Gesells.* 16:368-377. 1898.

BRUNSTEIN, A., Ueber Spaltungen von Glycosiden durch Schimmelpilze. *Beih. Bot. Centralbl.* 10:1-50. 1901.

KOHNSTAMM, P., Amylolytische, glycosidspaltende, proteolytische und cellulose-lösende Fermente in holzbewohnenden Pilzen. *Ibid.* 10:90-121.

⁹ DUCLAUX, E., Sur la nutrition intracellulaire. *Ann. Inst. Pasteur* 3:97-112. 1889.

grown mycelia were not suitable for the germination of spores, the spores used in the first cultures were germinated before inoculation. This method was extremely unsatisfactory, because the germinating spores could not be evenly distributed in the culture liquid. Moreover, it was found that spores germinated well in all the media used. Attempts to inoculate the flasks by means of the platinum wire also proved failures, because it was impossible to obtain an even distribution of the dry spores. The method finally adopted, which proved entirely satisfactory, was as follows: A stock-culture on beans, which was well covered with spores, was thoroughly shaken up with the liquid in which it was growing; the liquid was then poured on a screen of fine-meshed muslin in a funnel, and strained into a sterilized flask. This gave a liquid turbid with spores, most of which had been shaken apart and floated free in the liquid. Very little other material passed into the flask. For inoculation three drops of this liquid were dropped into each flask from a sterile pipette. This gave an abundance of spores, perhaps some thousands, which were uniformly distributed in the culture fluid.

The yield of dry material produced was used as an indicator of the effect of the food given. The yield was determined by adding 10^{cc} of a 10 per cent. solution of chemically pure hydrochloric acid to the culture to kill the growth and dissolve any precipitates that had been formed. After this the culture was filtered on a hard filter paper, from which the fungous material was washed into a tared Gooche crucible, and dried at 100–110° C. The variation in the temperature at which the yields were dried was not preventable, since no regulated drying oven was available. All the yields of each series were dried at the same time, however, so that they were subjected to the same conditions in drying. The different sets of each series are therefore comparable with each other.

TABLES AND EXPLANATIONS

In the following tables are given the results of the cultures, with the necessary explanations relating to each series. In the course of the work many hundreds of cultures were made before finally satisfactory details of manipulation were worked out, and for the purpose of determining the concentrations of the various substances that permitted

the best growth of the mold. None of these preliminary series is included in this report, although the facts gained from them served in a general way to substantiate the results of the other cultures.

Alcohol.—One table, made for the purpose of determining how long the minimum quantity of alcohol used in some of the cultures could provide sufficient food for an increase in growth, may not be without interest and is here given as *table I*. The yield is given in milligrams in all cases.

TABLE I

Each flask received 0.46^{gm} alcohol = concentration of 0.2 GM. per liter.

Time in days (24 h.)	1	2	3	4	5
1.....	0.0	0.1
2.....	1.4	1.8	1.9
3.....	13	14	15	16	17
4.....
5.....	38	39	43	53	58
6.....	59	61	65	79	92
7.....	80	91	95	104	110
8.....	118	119	120	122	124
9.....	118	121	124	128	128
10.....	110	113	114	115	120

All the cultures in each horizontal row are duplicates. An increase in weight took place up to the tenth day, after which there was a gradual loss, due to respiration.

TABLE II

Each flask received 0.69^{gm} alcohol = concentration of 0.3 GM. per liter.

Number	I. No acid	II. 0.004 N HCl	III. 0.004 N HNO ₃	IV. 0.004 N H ₂ SO ₄	V. Check. No organic compounds
1.....	53	90	106	113	4
2.....	57	108	120	116	
3.....	86	114	125	131	
4.....	89	115	127	139	
5.....	93	118	144	141	

TABLE III

Each flask received 0.46^{gm} alcohol = concentration 0.2 GM. per liter.

Number	I. No acid	II. 0.004 N HCl	III. 0.004 N HNO ₃	IV. 0.004 N H ₂ SO ₄
1.....	68	91	100	88
2.....	70	93	106	89
3.....	71	93	106	91
4.....	73	102	108	93
5.....	77	103	110	95

In about 200 preliminary cultures it was found that the addition of small quantities of acids such as HCl, HNO₃, and H₂SO₄ had a stimulating effect on the growth of *Penicillium*. This is only an instance of the general rule that poisonous substances act as stimulants when given in sufficiently dilute concentration. These two series were carried through with great care and uniformity, in order to get, if possible, a more accurate quantitative statement of the facts noted in the preliminary cultures. There is considerable individual difference in the cultures, as is noted by reading the figures in each column, the five cultures being duplicates of one another. Nevertheless, they are of the same general magnitude. The addition of mineral acids produces a decided stimulation of growth. The nitric acid in each case gave a greater stimulation than the hydrochloric. The sulfuric acid in one case (*table II*) gave a greater increase than hydrochloric, while in the other case (*table III*) its effect was about equal to that of the HCl. These observations agree with those made on the preliminary cultures. Many of the cultures seemed to show that nitric and sulfuric acids produced greater stimulation than hydrochloric acid. It was thought possible that HCl might begin to act as a poison at the concentration used, but a series of cultures (*table IV*) showed that the optimum for stimulation was much higher than 0.004*n*. Therefore HCl at that concentration cannot act as a poison, but is actually a milder stimulant than HNO₃, and perhaps also weaker than H₂SO₄. In addition to the usual mineral nutrients, the cultures in *tables II, III, and IV* were given 0.25^{gm} KCl per 100^{cc} of culture fluid, to avoid the introduction of a new ion, Cl, in the cultures to which HCl was added. None of the alcohol cultures produced spores.

TABLE IV

Each flask received 0.69^{gm} alcohol = concentration 0.3 GM. per liter.

Number	CONCENTRATION OF ACID								
	1	2	3	4	5	6	7	8	9
	0.000	0.002 <i>n</i>	0.004 <i>n</i>	0.006 <i>n</i>	0.008 <i>n</i>	0.012 <i>n</i>	0.016 <i>n</i>	0.024 <i>n</i>	Check
1.....	208	172	146	266	183	193	236	163	3.6
2.....	209	227	185	268	255	239	239	171	3.6
3.....	209	232	195	284	257	246	240	173	
4.....	214	239	266	285	260	255	246	181	
5.....	224	242	280	295	273	255	257	181	

Esters of alcohol with mineral acids.—The cultures with esters of alcohol with mineral acids gave the results expressed in the following tables. The actual quantity as well as the concentration of the organic substances in each flask are given in *tables V* and *VII* in the horizontal lines, and in *tables VI* and *VIII* at the head of the columns giving the yields for each set.

TABLE V

Numbers	Amount of $C_2H_5KSO_4$	Yield
I-10	0.82 ^{gm} (=0.1 GM. per l.)	none
II-20	1.64 ^{gm} (=0.2 GM. per l.)	none

TABLE VI

Number	0.82 ^{gm} $C_2H_5KSO_4$ (=0.1 GM. per l.) 0.69 ^{gm} C_2H_5OH (=0.3 GM. per l.)	0.82 ^{gm} $C_2H_5KSO_4$	0.41 ^{gm} $C_2H_5KSO_4$ (=0.05 GM. per l.) 0.69 ^{gm} C_2H_5OH	0.41 ^{gm} $C_2H_5KSO_4$	Check 0.69 ^{gm} C_2H_5OH
1.	152*	None	200	None	193
2.	184	None	210	None	
3.	207	None	214	None	
4.	219	None	228	None	
5.	219	None	238	None	

* Contaminated.

TABLE VII

Numbers	$C_2H_5NO_3$ *	Yield
I-10	0.46 ^{gm}	None
II-20	0.91	None

* The concentration of the ethyl nitrate is not stated, since only a part of the amounts given goes into solution in 50^{cc} of the culture fluid.

TABLE VIII

Number	0.23 ^{gm} $C_2H_5NO_3$ 0.69 ^{gm} C_2H_5OH (=0.3 GM. per l.)	0.46 ^{gm} $C_2H_5NO_3$ 0.69 ^{gm} C_2H_5OH	0.69 ^{gm} C_2H_5OH	Check. No organic matter
1.	103	52	68 117	2
2.	106	56	94 118	
3.	112	65	112 141	
4.	115	72	113 143	
5.	126	89	116 146	

The cultures of potassium ethyl sulfate (*table V*) had a barely visible film on the surface, similar to cultures to which no organic matter had been added. Microscopic examination showed that prac-

tically all the spores had germinated and had produced germ tubes 50 to 100 μ long. This is clear proof that the potassium ethyl sulfate is not poisonous in the concentrations used, but also that it is not a suitable source of carbon. This is more fully brought out in *table VI*. The cultures with potassium ethyl sulfate behaved as before, while those with sulfate to which alcohol was added gave a yield comparable with that obtained when alcohol alone was given. These cultures, like the alcohol cultures, remained pure white and produced no spores.

Ethyl nitrate in various concentrations ranging from 0.46^{gm} per 50^{cc} to 0.91^{gm} per 50^{cc} proved absolutely valueless as a source of carbon, although like potassium ethyl sulfate it did not inhibit germination at these concentrations. In a series in which the ethyl nitrate was given as the sole source of carbon and nitrogen, no growth took place. Neither is the fungus able to break up the nitrate by means of energy derived from alcohol, as *table VIII* shows, for the addition of ethyl nitrate failed to produce an increase of yield over pure alcohol. On the contrary, the higher concentration depressed the yield, showing that ethyl nitrate is mildly toxic. This is also shown by the fact that spores in cultures with ethyl nitrate germinated two to three days later than the alcohol cultures. The depression of yield may be due partly to the delay in germination, and partly to the toxic effect of the nitrate. The cultures produced no spores.

Ethyl acetate.—While the esters of alcohol with mineral acids proved valueless as a source of carbon for *Penicillium*, the ethyl ester of acetic acid in dilute solution is an efficient source of carbon; but in stronger solutions it becomes a poison, as appears from *tables IX* and *X*. The mineral solution used here was of the same composition as in the alcohol cultures.

TABLE IX

Each flask received 0.22^{gm} $\text{CH}_3\text{COOC}_2\text{H}_5 = 0.05$ GM. per liter.

Number	No mineral acid	0.004 N HNO_3	0.004 N H_2SO_4	0.004 N HCl
1.....	13	12	10	11
2.....	14	12	11	11
3.....	14	12	12	11
4.....	14	12	12	12
5.....	15	12	12	13

TABLE X

No.	I 0.88 ^{gm} CH ₃ COOC ₂ H ₅ (=0.2 GM. per l.) 0.69 ^{gm} C ₂ H ₅ OH (=0.3 GM. per l.)	II 0.88 ^{gm} CH ₃ COOC ₂ H ₅	III 0.44 ^{gm} CH ₃ COOC ₂ H ₅ (=0.1 GM. per l.) 0.69 ^{gm} C ₂ H ₅ OH	IV 0.44 ^{gm} CH ₃ COOC ₂ H ₅
1....	None	None	59	19
2....	None	None	74	20
3....	None	None	79	21
4....	None	None	89	22
5....	None	None	90	22

Ethyl acetate is easily soluble in water and forms a suitable source of carbon for fungi. It is poisonous, however, in comparatively low concentrations. The fungi in this medium grew for a time and then ceased growing, owing to the accumulation of acetic acid in the cultures. The addition of mineral acids had no very evident effect on the growth of the mold, but seemed to depress rather than increase the yield. It is important to note that the cultures containing alcohol in addition to ethyl acetate (*table X, col. III*) gave a yield about equal to that produced by alcohol alone (*table II, col. I*). It is probable that the alcohol, which is the better food, is largely responsible for this growth, and that the acetate was left largely intact. If it had been consumed in the usual way, the accumulation of acetic acid would have stopped the growth of the cultures. The cultures containing alcohol failed to fruit, while those containing only ethyl acetate fruited abundantly. The spores from sets I and II, *table X*, were killed, as no growth appeared when they were transferred to bean-stems.

Acetic acid.—Acetic acid in its free state forms an efficient source of carbon, but is so highly toxic that it must be used in extremely dilute solutions. Nevertheless, two series of cultures placed the fact of its assimilation beyond doubt. These are given here.

TABLE XI

Acetic acid in each flask 0.012^{gm} (=0.004*n*).

Number	No mineral acid	0.004 <i>n</i> H ₂ SO ₄	0.004 <i>n</i> HNO ₃	0.004 <i>n</i> HCl	Check
1.....	2	1	2.5	1	I
2.....	3	2	3	2	
3.....	3	3	3	2	
4.....	3	3	3.5	3	
5.....	3	3	4	3	

The addition of mineral acids seems to have no depressing effect, although the yields are so small that this would perhaps not be made evident. In the second series the weights were not determined, for the difference in growth is shown more strikingly by the appearance of the cultures than by the difference in weights. These cultures were made as follows:

TABLE XII

1-5.....	No acid	Bare trace of growth
6-10.....	0.024 ^{gm} per flask (=0.008 <i>n</i>)	Good growth forming thin flocculent film
11-15.....	0.048 ^{gm} per flask (=0.016 <i>n</i>)	No germination
16-20.....	0.072 ^{gm} per flask (0.032 <i>n</i>)	No germination

All the acetic acid cultures fruited, so that even the minute colonies were blue with spores.

Potassium acetate.—A large number of cultures was made with potassium acetate. The data from the most significant series of those are given here.

TABLE XIII

Mineral nutrients per 100^{cc} solution 1^{gm} NH₄NO₃, 0.5^{gm} KH₂PO₄, 0.25^{gm} MgSO₄, 0.25^{gm} KCl. Quantity and concentration of CH₃COOK per flask is given at the head of each column.

No.	I 0.245 ^{gm} CH ₃ COOK (=0.05 G.M. per l.)	II 0.40 ^{gm} CH ₃ COOK (=0.1 G.M. per l.)	III 0.98 ^{gm} CH ₃ COOK (=0.2 G.M. per l.)	IV 1.47 ^{gm} CH ₃ COOK (=0.3 G.M. per l.)
1....	31	41	40	40
2....	31	42	41	40
3....	32	42	41	41
4....	32	43	42	42
5....	32	43	42	51

TABLE XIV

The conditions of this series were the same as those for the preceding, except that no potassium chlorid was used in the mineral solution.

No.	I 0.245 ^{gm} CH ₃ COOK	II 0.49 ^{gm} CH ₃ COOK	III 0.98 ^{gm} CH ₃ COOK	IV 1.47 ^{gm} CH ₃ COOK
1....	30	26*	39	40
2....	31	40	40	41
5....	32	41	41	41
4....	32	41	41	41
5....	32	43	42	42

* Contaminated with a bacterium forming a green pigment.

TABLE XV

In this series the amount of CH_3COOK was 0.98gm per flask, but the magnesium content was varied.

Number	I	II
	0.5 ^{gm} MgSO_4 per 100 ^{cc}	1 ^{gm} MgSO_4 per 100 ^{cc}
1.....	54	5
2.....	55	14
3.....	55	17
4.....	55	20
5.....	56	37
6.....	56	39
7.....	56	41
8.....	57	47
9.....	57	55
11.....	58	64

The potassium acetate cultures show a remarkable uniformity of yield, which is independent of the concentration of the acetate, except in very dilute solutions and probably also in very concentrated solutions. The lowest concentration in *tables IX* and *X* is too dilute to allow the full development of the fungus under those conditions, but all the other concentrations give the same yield.

With the increase of magnesium in the cultures, the yield is increased but still remains uniform (*table XV, col. I*). A higher concentration of MgSO_4 becomes poisonous.

In the growth of all the potassium acetate cultures the medium becomes alkaline, showing that only the CH_3COO^- radicle is taken up by the fungus. The excess of KOH (or KHCO_3) results in the precipitation of NH_4MgPO_4 , thus keeping the culture neutral or only slightly alkaline for a time. When all the Mg is precipitated, the increased alkalinity prevents further growth. The addition of more Mg delays this period, and hence gives a greater yield. The yield is practically quantitative for a certain amount of Mg , and is independent of the amount of CH_3COOK .

The potassium acetate cultures brought out the striking difference in power of resistance to deleterious substances possessed by different spores from the same culture. This was also observed in other cases where deleterious concentrations of substances were used, but it was nowhere as evident as in the potassium acetate cultures. By the method of inoculation, it is likely that more than a thousand spores were sown in each flask. In the favorable media a large percentage

germinated so that the surface was covered from the beginning with a film of fungus. In the cultures containing the stronger solutions of CH_3COOK , sometimes less than a hundred colonies were formed, but these were able to grow with sufficient vigor to produce a yield equal to that of the flasks in which more spores had germinated.

All the potassium acetate cultures formed spores, but those in the concentration of 0.05 GM. per liter produced them most abundantly.

DISCUSSION

A general survey of these data shows that alcohol, acetic acid, and the substances from which the acetic acid radicle $\text{CH}_3\text{COO}-$ is easily derived are assimilated by *Penicillium glaucum*. In the case of alcohol the addition of mineral acids stimulates growth, but HNO_3 produces greater stimulation than HCl . The esters of alcohol with mineral acids are valueless as a source of carbon, and their lack of nutritive value is not due to any toxic properties. The substances which possess the greater food value among the foregoing are, in general, those which are readily oxidized. To what extent will these data enable us to correlate the mode of assimilation of these compounds with the known chemical reactions of the substances?

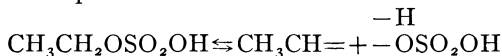
The first possibility that presents itself is that alcohol enters into combination with substances in the protoplasm by virtue of its dissociation into ethylidene and water



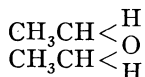
which, as NEF¹⁰ has shown, takes place completely at about 650° C. At ordinary temperatures dissociation into ethylidene and water takes place to a very slight extent, probably less than 0.01 per cent. The dissociation, however, is enormously increased by a combination of alcohol with other substances, as with metals or mineral acids, and also by the action of enzymes and other catalytic agents. The alcohols are dissociated to so great an extent at ordinary temperatures that they burn spontaneously in the air. Ethyl nitrate dissociates at about 200° C. (?) and potassium ethyl sulfate at 250° C. Ethyl sulfuric acid and ethyl sulfate dissociate at low temperatures, so that ether formation begins in a mixture of alcohol with a little sulfuric acid at

¹⁰ NEF, J. U., On the fundamental conceptions underlying the chemistry of the element carbon. Journ. Am. Chem. Soc. 26:1549-1577. 1904.

95° C. The reaction is due, as NEF¹¹ has shown, to the absorption of water by the bivalent carbon of the ethylidene particles. The reaction may be represented as follows. The dissociation of ethyl sulfuric acid takes place thus:



The sulfuric acid is regenerated and reacts with more alcohol to give ethyl sulfuric acid, or ethyl sulfate, while the ethylidene decomposes water to form ether thus:



If, in the first steps of assimilation, alcohol enters into combination with some substance of the protoplasm to form an unknown compound, represented by $\text{CH}_2\text{CH}=\text{Ppm}$, then we should expect substances which increase ethylidene dissociation to affect the rapidity of assimilation. It is immaterial whether the alcohol is first elaborated into sugar, glycerin, or some other substance, before it becomes a part of the protoplasm, or whether it is directly taken up by the permanent constituents of the cell. In either case it must combine with some substance of the cell, and this combination must be regarded as the first step in assimilation.

An examination of the cultures (*tables II, III*) shows an increase in growth due to the addition of sulfuric acid, which, even in dilute solutions, would to some extent combine with alcohol and dissociate into ethylidene; but an equal increase is obtained by the addition of hydrochloric acid, and nitric acid shows an even greater increase in most cases; yet neither hydrochloric nor nitric acid combines with alcohol when mixed with it. This would indicate that stimulation by the acids is not due to any dissociating effect on the alcohol. Furthermore, if assimilation took place by direct combination of ethylidene with some substance of the cell, then bodies which dissociate very easily should be most rapidly assimilated. We find, however, that potassium ethyl sulfate and ethyl nitrate, which dissociate to a high degree, are valueless as sources of carbon when given alone, and in all probability cannot even be utilized by means of energy derived

¹¹ NEF, J. U., Dissociationsvorgänge bei den Alkyläthern der Salpetersäure, der Schwefelsäure, und der Halogenwasserstoffsäuren. *Liebigs Annalen* 318:1-57. 1901.

from alcohol when it is given together with the esters. This would seem to argue against the direct absorption of ethylidene by the protoplasm or cell constituents.

Another possibility to be considered is the oxidation of alcohol to acetaldehyde or even to acetic acid. This view becomes the more probable on account of the ease with which alcohol is known in many instances to be oxidized by organisms. This view would perhaps explain also the increased stimulation of nitric acid over hydrochloric acid, on the basis of the oxidizing power of nitric acid. Oxidation to acetic acid, if it takes place at all, does not proceed to such an extent that the acid accumulates in the cultures.

To determine whether acetic acid was accumulated in the cultures, 10 flasks, with alcohol as an organic food, were inoculated in the usual way. After a vigorous growth took place, the culture solution was poured off, and after rinsing was replaced by a 0.3 GM. solution of alcohol. After further 6 days this liquid was tested for acetic acid, but none could be detected. DUCLAUX also was unable to observe the formation of acetic acid from alcohol by *Aspergillus*, but mentions the fact that alcohol was assimilated with the intermediate formation of oxalic acid. I was unable to show the presence of oxalic acid in the cultures of *Penicillium*, nor is it likely that this is formed. If alcohol is assimilated by way of acetic acid the oxidation takes place entirely within the cell.

Another fact gained from the ethyl acetate cultures (*table X*) speaks against the oxidation of alcohol to acetic acid. From the general principles resulting from the work on selective power of organisms by PASTEUR, DUCLAUX, and PFEFFER, we know that when two foods of different nutrient value are given to a plant, the one most readily assimilated is used, often to the exclusion of the other. Although the experiments with ethyl acetate have not been carried as far as might be desirable, there is some evidence that acid accumulates in the cultures, consequently that the alcohol radicle is more readily absorbed than the acid radicles. This, it would seem, would not take place if it was first necessary to oxidize the alcohol to acetic acid. If any oxidation takes place it is probable that this stops with acetaldehyde, from which the formation of sugars can proceed. It would then be probable that the acetic acid in all cases was reduced

to the aldehyde. This would account for the greater ease of assimilation of alcohol, since the oxidation of alcohol takes place more readily than the reduction of acetic acid.

The cultures of acetic acid and potassium acetate require no discussion, since it is evident, especially from the acetate cultures, that it is the acetate ion that is assimilated. This ion was assimilated, so far as could be determined, with much more difficulty than alcohol, which would be in accord with the belief that the acid must be reduced to aldehyde.

INCIDENTAL OBSERVATIONS

As in any long series of cultures, some facts were observed in this study, which, while not directly concerned with the work, may be of sufficient interest to be worth noting.

DUCLAUX is responsible for the belief, current in textbooks, that some substances which permit vigorous growth of mycelia are not suitable for the germination of spores. Alcohol is one of the substances mentioned by him. Contrary to this statement, it was found that alcohol was not only favorable for growth, but also permitted abundant germination of spores. From the preliminary cultures it seems probable that all concentrations which will permit growth will also permit germination.

In many other instances substances failed to allow mycelia to develop and yet were not detrimental to germination, e. g., $C_2H_5KSO_4$. This whole subject deserves further investigation, with careful study of the effect of different concentrations on growth and germination.

An interesting observation was made on the alcohol cultures, namely, that none of them produced spores during the growth of the cultures. It is difficult at present to offer an explanation of this fact. The fungus grew more vigorously in the alcohol cultures than in any others, and as far as could be observed all the external conditions necessary for the production of spores were present. The mycelia formed dense white mats on the culture liquid, and innumerable hyphae grew into the air—a condition which usually leads to the production of spores—yet none of the aerial hyphae bore spores. It would seem as if this strain of *Penicillium* was unable to manufacture all of the compounds necessary for spore formation from alcohol alone;

yet such an explanation is improbable when we remember that other compounds easily derived from alcohol, e. g., acetic acid, ethyl acetate, and potassium acetate, furnish material for the development of the fungus and the production of spores. It is also possible that the suppression of spores is due to some deleterious action of the alcohol in the culture fluid, although it is difficult to see how a substance can at one and the same time act as a food of high nutrient value and as a poison. At first sight the cultures containing both ethyl acetate and alcohol would seem to bear out the view that alcohol was deleterious to spore-formation, for cultures with ethyl acetate alone fruited, while those containing alcohol also failed to fruit. In such cultures, however, it is probable that the more nutritious alcohol was absorbed first, and largely to the exclusion of the acetate. Further work is necessary to give a complete explanation of this unusual phenomenon. It may be found that all strains of *Penicillium* do not act alike in this respect.

Another fact, brought out in the course of this investigation, was the great individual difference of resistance of spores to deleterious agents. In the lower concentrations of all substances favorable for growth, practically all spores germinated, forming a dense matlike growth over the surface of the culture fluid. When, with increase of concentration, the substance becomes deleterious, germination and growth are not stopped abruptly, but the number of colonies becomes fewer and fewer until the final concentration is reached, where germination of even the most resistant spores is inhibited. In most cases, where only a few colonies were formed, these grew with unusual vigor, so that the total weight of the culture was often as great as that of the cultures of lower concentrations.

This was especially well shown in the series with potassium acetate, *tables XIII--XV*. Here comparatively few spores germinated in the higher concentrations, forming isolated floating colonies. Yet the yield from these was equal to the yield from other flasks which were uniformly covered. This seems to indicate that the mycelium from the more resistant spores continues itself to be more vigorous throughout life, although it is not impossible that the belief of *DUCLAUX* holds true here; that is, while the concentration in any given case may be injurious to germination, it does not interfere with later development.

The spores which are able to germinate then have the whole of the nutrient solution at their disposal and make more vigorous growth.

In conclusion I wish to express my thanks to Professor CHARLES R. BARNES, under whom this work was carried on; and to Professor J. U. NEF of the Department of Chemistry for many helpful suggestions relating to the chemical aspects of the work.

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